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The antimicrobial potential of *Lactobacillus acidophilus* on pathogenic bacteria causing diarrhea

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Abstract

Object: *Lactobacillus acidophilus* is a nonpathogenic member of gastrointestinal tract and it is widely used in fermented dairy products. This study aimed to assess the antimicrobial potential of two strains of *L. acidophilus* on some pathogenic bacteria frequently causing diarrhea or gastroenteritis.

Methods: The antibacterial activity cell free supernatant (CFS) of two control standard strains of *L. acidophilus (L. acidophilus-la5* and *L. acidophilus* against five control standard strains of bacteria causing diarrhea; Enterotoxogenic *Escherichia coli (*ETEC), Enterohaemorrhagic *E. coli* O_{157}:H_7(EHEC) *Salmonella typhimurium Shigella flexeneri* and *Staphylococcus aureus* were determined using agar well diffusion method. The sensitivity of the pathogenic bacteria to the CFS of each *L. acidophilus* in relation to time was determined by standard plate count .The antibiotic susceptibility tests of 20 antibiotics against tested organisms with and without CFS were assessed by disc diffusion method. The minimum inhibitory concentration (MIC) of ciprofloxacin with and without CFS was determined by tube dilution method.

Results: Both *Lactobacilli* strains decrease the colony count of tested strains by more than 90% after 60 min contact time. Both *Lactobacilli* strains significantly improve the antibacterial effect of tested antibiotics against ETEC, *S. typhimurium* and *S. aureus*, and *S. flexeneri* (*P< 0.05)*. The MIC of ciprofloxacin alone against all tested strains was 15.625 µg/ml, while when combined with both *Lactobacilli* CFSs, the MIC decreased significantly to 0.488 µg/ml for ETEC, *S. typhimurium,* and *S. flexeneri* and to 0.977 µg/ml for EHEC and *S. aureus (P= 0.000)*.

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Conclusion: living *L. acidophilus* strains could be used in prevention and treatment of diarrhea caused by certain bacterial pathogens, either in fermented milk/ yoghurt or as mediations.

Key words: Probiotic, *L. acidophilus,* Bacterial Diarrhea

Introduction

Infective bacterial diarrhea is a global health problem especially in young children in developing countries with rotavirus is the most common identified pathogen [1]. Many bacterial species were implicated as a cause of infective diarrhea as *Salmonella* spp., *Campylobacter* spp., Shiga toxin-producing *E. coli* O157:H7 strain, *Shigella* spp., *Vibrio* spp.; and *Yersinia* spp. Other diarrheagenic *E. coli* in particular enterotoxigenic *E. coli* and enteroaggregative *E. coli* are increasingly being reported as causes of acute diarrhea [2].

Oral rehydration solution and antimicrobials are the main treatments for acute diarrhea. However, when diarrheal patients were given probiotics prior to or during hospitalization, a reduction in the frequency of diarrheal symptoms has been reported in both adults and children [1, 3].

Probiotics are preparations of living bacteria and yeasts that possess a beneficial health effect when administrated in adequate amounts [1, 4].They have been extensively studied for their beneficial effects in preventing and treating many conditions, including the treatment of lactose intolerance, traveller's diarrhea and the prevention and treatment of hospital acquired diarrhea[5, 6]. Previous studies have evaluated the effect of *Lactobacillus rhamnosus* on colonization of rotavirus and found a potential antimicrobial effect against it [7]. It has been reported that most probiotics are well tolerated with rare adverse effect and can be safely used in patients with underlying chronic diseases or in those on immunosuppressive therapy [1].

Lactic acid bacteria are a group of Gram-positive bacilli and cocci occurring naturally in gastrointestinal tract, plants and fermented foods, such as dairy products, meat and alcoholic beverages [4]. Most probiotics commercially available today belong to the genera *Lactobacillus* and *Bifidobacterium*. They are the most important group of microorganism used in food fermentations. They inhibit food spoilage and pathogenic bacteria by producing antimicrobial substances such as lactic acid, hydrogen peroxide and bacteriocins [8]. Several mechanisms by which probiotics *in vivo* mediate their health benefits in the host; first, certain probiotics have antimicrobial activity and can exclude or inhibit pathogens; second, they can enhance the intestinal epithelial barrier; third, probiotic bacteria are believed to modulate the host immune response [4].

The aim of this study was to investigate the antimicrobial potential of two *L. acidophilus* strain on pathogenic bacteria frequently causing diarrhea or gastroenteritis with and without antibiotics.

Materials and Methods

The test organisms

Five standard strains of diarrhea causing bacteria; Enterotoxogenic *E. coli (*ETEC) (ATCC 25922), Enterohaemorrhagic *E. coli* O_{157}:H_7 (EHEC) (ATCC 51659), *S. typhimurium* (ATCC 25566), *S. flexeneri*(ATCC

29903, CCM 4422) and *S. aureus* (ATCC 13565) were used in this study. The test organisms were grown in Brain Heart Infusion broth (Oxoid Ltd., Basingstoke, UK) and incubated at 35°C for 18 hrs. The concentrations of the organisms were determined by spectrophotometric method and standardized for all organisms at 10^3 cfu/ml [9].

Preparation of cell-free *L. acidophilus* culture supernatants

Two standard strains of *L. acidophilus (L. acidophilus-la5* (L1) and *L. acidophilus* (ATCC 4356, DSM 20079) (L2) were grown in MRS broth (Oxoid Ltd., Basingstoke, UK) at 35˚C for 18 hrs and adjusted to 0.5 *McFarland.* The cultures were centrifuged at 10000xg for 15 min and the resulted supernatant was designated crude cell –culture free supernatant (*CFS*). These supernatants were used immediately or stored at -20˚C until needed for antibacterial activity [10].

Determination of the antibacterial activity of *L. acidophilus*

The antibacterial effect of L1 and L2 was investigated by the following methods:

1. *Agar well diffusion method (Qualitative inhibitory effect of CFS of lactobacilli strains).* Agar well diffusion method was used as described by Wolf and Gibbones [11]. Briefly the freshly prepared inoculum (10^8 CFU/ mL) was swabbed all over the surface of the Muller Hinton plate (Oxoid Ltd., Basingstoke, UK) using sterile cotton swab. Five wells were bored in the medium with the help of sterile cork-borer having 5-mm diameter and were labeled properly. Then 50 µL of each *CFS* was added to each well, 50 µL of sterile nutrient broth was added to a well as a control. The experiment was done in triplicate. All plates were incubated at 37°C for 24 hours. After incubation, zones of inhibition (IZ) were measured.
2. *Standard plate count (Quantitative inhibitory effect of CFS of lactobacilli strains):* Equal amounts of *CFS* of each *L. acidophilus* and each test organism at 10^3 CFU/ml were mixed in a sterile flask. The mixture was stirred gently. 100 µl was immediately transferred to nutrient agar (0 contact time) and incubated at 35°C for 18-24 hours. 100 µl sterile MRS broth was used as a negative control. This procedure was repeated at intervals of 10 minutes up to 60 minutes (0, 10, 20, 30,40,50,60 minutes). Standard plate count was evaluated after incubation [9].
3. *Antibiotic susceptibility test with and without lactobacilli.* This test was done by using Kirby-Bauer disc diffusion method. Muller-Hinton plates were inoculated by swabbing the tested organism after dilution of 10^8 cfu/ml organisms with equal amount of nutrient broth (Oxoid Ltd., Hampshire, UK) onto the surface of agar plates. Antibiotic discs were applied and plates were incubated for 24 hours at 35˚C. The antibacterial activities of antibiotics were assessed by measuring the inhibition zone (IZ) in mm. Equal amounts of CFS of each *L. acidophilus* and each test organism at 10^8 cfu/ml was mixed in a sterile flask at 0 contact time and the previous steps were performed and the results of both of tests were compared [12]**.** The following antibiotics discs (Oxoid Ltd., Hampshire, U K) were used: Rifampicin (30 µg), Norfloxacin (10 µg), Cefepime (30 µg), Cefoperazone (30 µg), Chloramphenicol (30 µg), Ampicillin/Sulbactam (20 µg), Amoxicillin (10 µg), Ciprofloxacin (5 µg), Gentamycin (µg), Cefotraxione (CRO) with 30 µg disc content, Sulphamethoxazole/Trimethprim (25µg), Doxycyline (30 µg), Erythromycin (15 µg), Amikacin (30 µg), Amoxicillin/Clavulanic acid (30 µg), Vancomycin (30 µg), Meropenem (10 µg), Clindamycin (10 µg), Tobromycin (30 µg), Cefotaxime (30 µg).

4. *Antibacterial effect of L. acidophilus on minimum inhibitory concentration (MIC) of ciprofloxacin against diarrhea causing bacteria***.** MIC of ciprofloxacin on tested organisms with and without lactobacilli supernatant were performed using macro-tube dilution method. MICs of ciprofloxacin alone and MICs of CFS alone: for each organism was determined as described by Clinical and laboratory standards institute [13]**.** For determination of MIC of ciprofloxacin with lactobacilli, serial two-old dilution of ciprofloxacin in supernatant of each *Lactobacillus* CFS was prepared and inoculated with 25 µl of 10^8 cfu/ml of test organism to each tube, mixed well and incubated at 35˚C for 18 hrs

Statistical analysis

All statistical analysis were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows. The independent samples *t*-test was used for comparison between the antimicrobial effect of antibiotic with and without L1 and L2. *P* value ≤ 0.05 was considered the cut-off value for statistically significance and *P* value ≤ 0.001 was considered highly significant.

Results

Both *Lactobacilli* (L1 and L2) strains dramatically reduced the colony count of tested strains; the decrease is directly proportional to the time of contact between organism and lactobacilli supernatants (**Figure 1**). We found more than 50% reduction in colony count of all tested organisms after ten minutes of contact with supernatants of both *Lactobacilli,* except for *S. aureus* it needs 30 min. The maximal effect was on ETEC. L2 needs more contact time than L1except for *S. aureus*. No significant difference between L1 and L2 supernatants in their antibacterial effect against tested organisms (*P* ≥ 0.05).

The inhibitory diameter of L1 and L2 against tested organisms are shown in **Table 1**. *S.typhimurium* is apparently the most affected organism although the differences between the two diffusion diameters of L1 and L2 are non-significant (*P* > 0.05).

Table 1. Inhibition zone (mm± SD) of L. acidophilus (L1 and L2) on tested organisms

Tested organisms	*L. acidophilus-la5* (L1)	*L. acidophilus* ATCC 4356(L2)
EHEC	12±1	12±2
ETEC	16±2	14±1
S. typhimurium	16±1	19±2
S. flexeneri	15±1	12±1
S. aureus	12±2	14±1

Table 2 shows both Lactobacilli strains significantly improve the antibacterial effect of tested antibiotics by disc diffusion method against ETEC, *S. typhimurium* and *S. aureus*, and *S. flexeneri* (*P <0.05)*. It seems that the best effect of L1 and L2 were on amoxicillin/clavulanic acid, Cefoperazone, clindamycin, erythromycin, rifampicin and vancomycin.

L1 and L2 potentiate the antibacterial effect of ciprofloxacin on Enterotoxogenic *E. coli,* Enterohaemorrhagic *E. coli* O157:H7, *S. typhimurium*, *S. flexeneri* and *S. aureus* as the MIC of ciprofloxacin alone against all tested strains was 15.625 µg/ml, while when combined with L1 or L2 supernatant, the MIC significantly decreased to 0.48µg/ml, for ETEC, *S. typhimurium,* and *S. flexeneri* and to *0.977* µg/ml, for EHEC and *S. aureus (P= 0.000)* **(Figure2)**.

Ciprofloxacin is effective against all bacteria tested, and showed almost similar results (inhibi-

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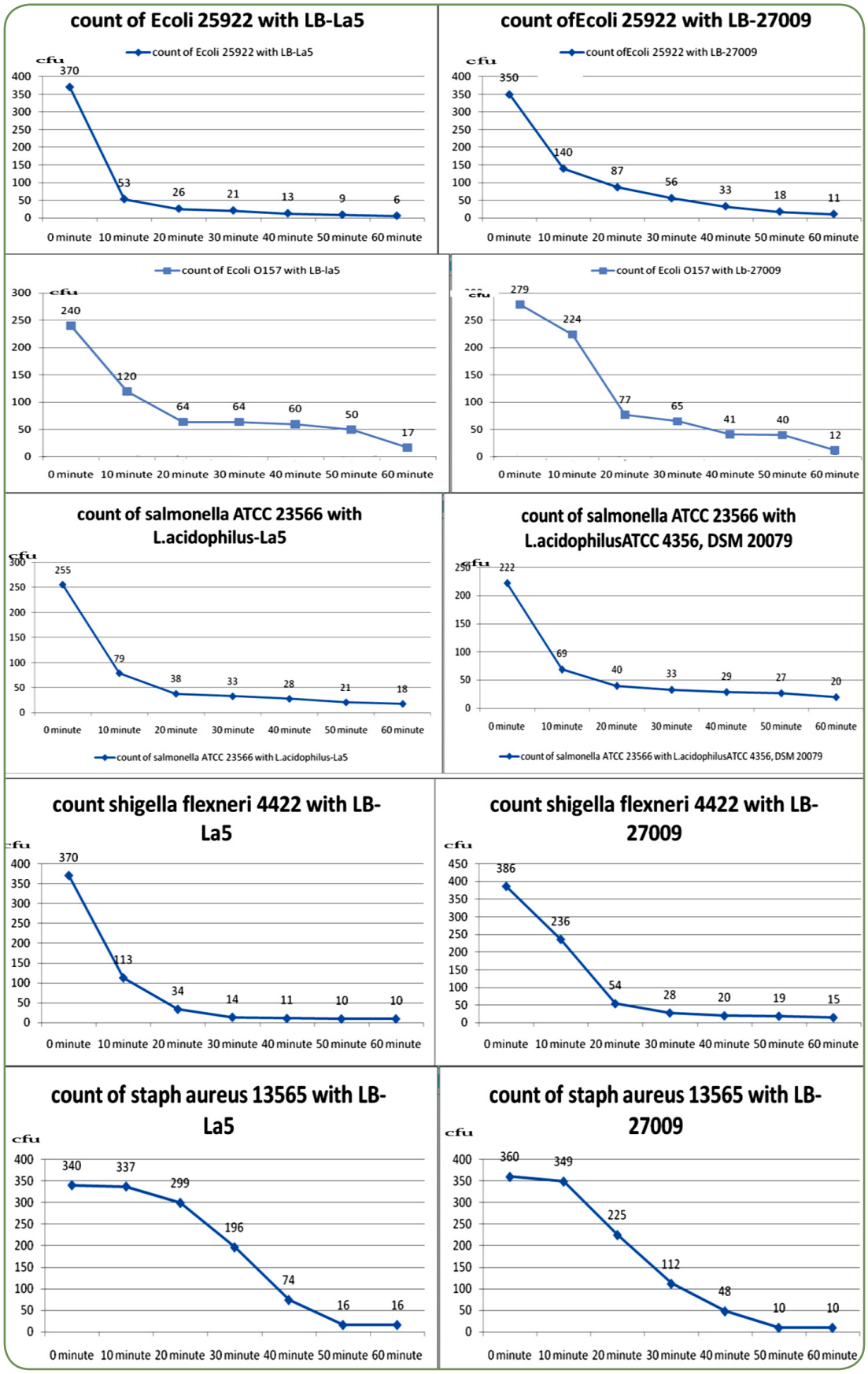

Figure 1. Time kill curve of *L. acidophilus* on tested organisms:

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Table 2. The antimicrobial effect of different antibiotics on tested organisms with and without L1 and L2 by disc diffusion methods (mm)

Antibiotic	EHEC	EHEC+L1	EHEC+L2	ETEC	ETEC+L1	ETEC+L2	S. typhimurium	S. typhimurium+L1	S. typhimurium+L2	S. flexeneri	S. flexeneri+L1	S. flexeneri+L2	S. aureus	S. aureus +L1	S. aureus+L2
Amoxicillin	0=R	0=R	0=R	0=R	0=R	0=R	12=R	13=R	12=R	18=I	19=I	18=I	9=R	12=R	13 =R
Ampicillin/Sulbactam	0=R	0=R	0=R	0=R	0=R	0=R	0=R	10=R	0=R	15=S	18=S	24=S	17=S	23=S	22=S
Amoxicillin/Clavulani	12=R	14=I	15=I	18=R	21=R	19=R	17=I	21=S	21=S	15=I	16=I	15=I	10=R	13=R	16=R
Cefepime	15=I	S=23	25=S	20=S	26=S	27=S	0=R	10=R	11=R	0=R	0=R	0=R	16=I	16=I	17=I
Cefoperazone	20=I	21=S	21=S	23=S	28=S	28=S	25=S	28=S	25=S	20=I	25=S	35=S	15=R	16=I	15=R
Cefotaxime	24=S	40=S	38=S	17=I	19=I	19=I	5=R	12=R	12=R	24=S	40=S	40=S	23=S	23=S	23=S
Cefotraxione	20=I	25=S	25=S	23=S	31=S	31=S	12=R	20=I	12=R	23=S	37=S	40=S	23=S	26=S	27=S
Vancomycin	0 = R	19=S	0=R	0=R	0=R	12=R	0=R	22=S	25=S	17=S	20=S	20=S	16=S	17=S	18=S
Meropenem	23=S	25=S	26=S	35=S	43=S	44=S	36=S	48=S	44=S	21=S	23=S	32=S	23=S	23=S	24=S
Clindamycin	0=R	20=I	25=S	0=R	9=R	0=R	0=R	46=S	23=S	35=S	44=S	40=S	21=S	28=S	26=S
Chloramphenicol	28=S	28=S	31=S	26=S	34=S	44=S	28=S	32=S	32=S	25=S	35=S	25=S	29=S	30=S	30=S
Rifampicin	13=R	21=S	16=R	17=I	48=S	43=S	16=R	38=S	43=S	10=R	20=S	32=S	R =15	40=S	38=S
Norfloxacin	30=S	30=S	32=S	35=S	40=S	40=S	32=S	40=S	39=S	30=S	33=S	40=S	30=S	33=S	32=S
Ciprofloxacin	31=S	31=S	32=S	35=S	43=S	41=S	35=S	38=S	42=S	30=S	46=S	46=S	32=S	34=S	33=S
Gentamycin	20=S	20=S	21=S	25=S	28=S	37=S	21=S	35=S	28=S	17=S	25=S	30=S	21=S	22=S	22=S
Tobromycin	16=S	22=S	25=S	15=S	20=S	21=S	19=S	28=S	30=S	21=S	24=S	21=S	18=S	20=S	20=S
Doxycyline	18=I	31=S	23=S	16=S	23=S	25=S	23=S	43=S	38=S	16=S	24=S	28=S	20=S	32=S	30=S
Erythromycin	0= R	18=I	0=R	10=R	38=S	38=S	0=R	25=S	35=S	11=R	11=R	25=S	13=R	32=S	30=S
Amikacin	21=S	26=S	26=S	21=S	27=S	33=S	21=S	24=S	23=S	20=S	31=S	30=S	20=S	24=S	22=S
Sulphamethoxazole/T	27=S	32=S	32=S	25=S	31=S	34=S	27=S	35=S	35=S	24=S	36=S	46=S	30=S	32=S	30=S
P value	-	0.054	0.19	-	0.08	0.04*	-	0.003*	0.014*	-	0.036*	0.033*	-	0.043*	0.046*

R: Resistant, S: Susceptible, I: Intermediate resistant, *significant

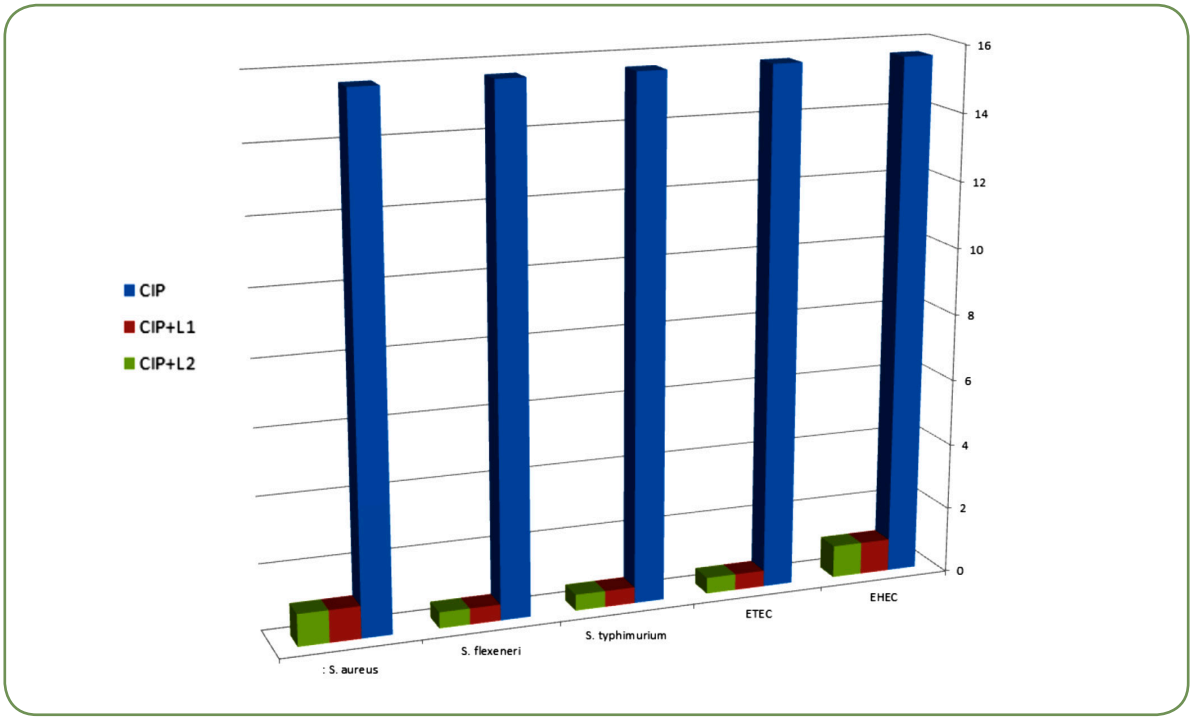

Figure 2. MICs of Ciprofloxacin (µg/ml(with and without L. acidophilus (L1 and L2) against tested organisms

tion zone) with or without lactobacilli strains (L1 and L2) as shown in **Table 2 and Figure 2**. However, their MICs are highly different according to the method used and the difference in dilution factors. The inhibition zones of ETEC, *S. typhimurium* and *S. flexeneri* are considerably increased in presence of L1 and L2 with ciprofloxacin.

Discussion

We have studied the effect of *Lactobacillus acidophilus* on diarrhea causing bacteria; *E. coli, S. typhimurium, S. flexeneri* and *S. aureus.* The *Lactobacilli* have been shown to possess inhibitory activity toward the growth of pathogenic bacteria. Our results revealed that the count of *E. coli* O157:H7 decreased after 60 minutes from 240 to 17 cfu after mixing with *LB-La5* (L1) and from 279 to 12 cfu after mixing with *LB*- ATCC 4356 (L2). As regarding the effect of L1 on control *E-coli* strain, the count of the latter deceased from 370 to 6 cfu after 60 minutes, while the count deceased from 350 to 11 cfu after 60 minutes with L2. In agreement with these results, Ogawa *et al*. [14] had documented the bactericidal activities of *L. acidophilus* against *E.coli.*

Abdel-Daim *et al*.[15] studied the probiotic potential and antagonistic activities of 32 *Lactobacillus* isolates against *S. typhi* and found that twelve *Lactobacillus* isolates could protect against *S. typhi* infections by interference with its growth and its virulence properties, such as adherence, invasion, and cytotoxicity. These results are nearly similar to the results of the current study since we found decease in the count of *Salmonella* (from 255 to18 cfu and from 222 to 20 cfu) after 60 minutes mixing with CFS *of lactobacilli* (LB-La5) and *lactobacilli* (LB-ATCC 4356), respectively.

This study revealed that the count of *S. flexneri* decreased to 10 cfu after contact with *Lactobacillus (LB-La5)* for 60 min, and to 15 cfu after mixing with *LB*- ATCC 4356 for 60 min. The study of Zhang *et al.* [16] has documented that lactobacilli strongly inhibit the gastrointestinal pathogen *Shigella.*

The present study found that the count of *S.aureus* decreased from 340 to16 cfu and from 360 to 10 cfu , respectively after mixing with *(LB-La5)* and *LB*- ATCC 4356, over one hour. Dicks and Botes [17] had reported that hydrogen peroxide produced by some strains of Lactobacilli, effectively inhibits *Staphylococcus aureus,* and *L. acidophilus* isolated from humans due to production of bacteriocin and non-bacteriocin antimicrobial substances which are active (both *in vitro* and *in vivo*) tests against Gram-positive and Gram-negative pathogens. Similar results were previously obtained by the study of Bassyouni *et al*. [4], where 8 strains of Lactobacilli isolated from different dairy products showed antimicrobial effects on clinical isolates of *E.coli, Staphylococcus spp., Salmonella spp. Micrococcus spp.* using well diffusion method and with inhibition zone ranging from 13 to 25 mm.

However, the result of this study disagree with Szymanski *et al*. [18] who has indicated that the effect of Lactobacilli was limited to the treatment of *rotavirus* induced diarrhea in children but not to the treatment of diarrhea of other etiology. Another study conducted by Szajewska *et al*. [19], reported also limitation of the therapeutic effects of probiotics on watery diarrhea and viral gastroenteritis but not against invasive bacterial diarrhea in children.

Synergistic effect of probiotics and antibiotics have been studied since 1990. Tomioka *et al*. [20] has investigated the effect of ofloxacin combined with *Lactobacillus casei* against *Mycobacterium fortuitum* induced infection in mice. They found that multiple injections of ofloxacin (subcutaneous or oral) in combination with a *Lactobacillus casei* preparation LC9018 (subcutaneous), in mice infected intravenously with *Mycobacterium fortuitum* led to the following; a marked delay in the incidence of spinning disease, a lowered incidence of gross renal lesions, and an increase in the rate of elimination of organisms from the kidneys. Their result in-

dicates synergism in the therapeutic efficacy of the two agents. We have studied the effect Lactobacilli (L1and L2) in association with 20 antibiotics using disc diffusion method and found that both Lactobacilli significantly improve the antibacterial effect of tested antibiotics against ETEC, *S. typhimurium* and *S. aureus*, and *S. flexeneri* (*P< 0.05)*. Similar results were reported by Ruiz *et al*. [21] using selected *Lactobacillus fermentum* strain L23 and *L. rhamnosus* strain L60 as an alternative treatment to prevent or treat urogenital infections. Their antimicrobial activity tests of L23 and L60 were performed by a disc diffusion method against 207 bacterial isolates from female presenting with urinary or genital infections. Their results showed 100% of the clinical isolates were susceptible to the antimicrobial substances produced by L23 and L60, and the selected lactobacilli produced larger inhibition zones when compared to several antibiotics commonly used for treating these infections. Synergistic interactions and indifferent interactions were recorded in 68.6% and 31.4% of the cases, respectively, while no antagonistic interactions were observed. Also, Hussein *et al*. [22] found that *L. acidophilusis* specifically antagonizes *H. pylori* and enhances antibiotic therapy and its culture supernatants inhibit ulcer formation.

Fluoroquinolones are generally considered as the drugs of choice for the empirical treatment of diarrhea in adults, they are active against most of the common intestinal pathogens with excellent tissue and intracellular penetration, and achieve high fecal concentrations and have a good safety profile in adults [23]. Therefore, we investigated the effect of *L. acidophilus* with presence of ciprofloxacin against diarrhea causing bacteria *in vitro*. The MIC of ciprofloxacin alone against all tested strains was 15.625 µg/ml, while when combined with both *lactobacilli* supernatants, the MIC decreased significantly to 0.488 µg/ml for ETEC*, S. typhimurium* and *S.flexeneri* and to 0.977 µg/ml for EHEC and *S. aureus* (P= 0.000). The overall results of this study are similar to results of Kaur and Sharma [24], which evaluated the synergism between conventional antibiotics and the cell-free supernatant (CFS) of vaginal *Lactobacillus crispatus*156 against *P. aeruginosa* MTCC 741 by checkerboard titrations. The used CFS succeeded to increase the activities of ciprofloxacin, moxifloxacin, and streptomycin , and it has decreased the MIC of ciprofloxacin by 30 times and MICs of both moxifloxacin and streptomycin by 8 times.

In conclusion, this study suggests that *L. acidophilus* can be used as an alternative therapy in treatment of different forms of infective bacterial gastroenteritis/diarrhea by its natural source in fermented milk and yoghurt or as mediations. The combined therapy of *L. acidophilus* with ciprofloxacin in treatment of infective gastroenteritis will help to decrease the required dose of ciprofloxacin and subsequent its potential side effects on intestinal bacterial flora.

Conflict of Interest

The authors declare that they have no conflict of interest

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